American Journal of Hematology 51:324–327 (1996)

Heteroduplex Analysis in Hemophilia B: Detection of Two Novel Factor IX Gene Mutations

M. Catherine Driscoll, Agnes Chu, and Margaret W. Hilgartner
Department of Pediatrics, Division of Hematology-Oncology, New York Hospital-Cornell Medical Center, New York, New York

Heteroduplex analysis of polymerase chain reaction (PCR)-amplified factor IX (FIX) sequences in eight hemophilia B pedigrees localized the causative hemophilia mutation to a single exon in each case. Subsequent PCR-based direct DNA sequence analysis identified two novel FIX mutations and six recurrent mutations. Three of the eight pedigrees represent sporadic hemophilia B, and direct mutation analysis facilitated hemophilia carrier diagnosis in each case. © 1996 Wiley-Liss, Inc.

Key words: FIX gene mutation, hemophilia B, heteroduplex analysis

INTRODUCTION

Hemophilia B is an X-linked recessive bleeding disorder due to deficiency of coagulation factor IX (FIX), occurring at a frequency of 1/23,000 males. The FIX gene locus, at Xq27, is 34 kilobases (kb) long with eight exons coding for 415 amino acids [1]. The 1994 hemophilia B data base catalogues 1,142 patient entries, of which 476 are unique molecular events [2]. The identification of FIX gene mutants has been escalated by the use of screening techniques based on the polymerase chain reaction (PCR) coupled to direct PCR-based DNA sequence analysis. The most commonly used screening methods include single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DDGE), and heteroduplex (HD) analysis [3–5]. These techniques are based on the altered migration of mutant single-strand DNA (SSCP), double-strand DNA (DDGE), and mixed heteroduplexed DNA strands (HD) in polymer gels. The high sensitivity of HD analysis in detecting FIX gene mutations in hemophilia B has already been demonstrated [6]. Since HD analysis is a nonradioactive method requiring minimal manipulation of PCR-generated DNA, we elected to perform an initial screen for FIX gene mutations using this technique.

MATERIALS AND METHODS

Patients

Eight Caucasian women who had requested studies for hemophilia B carrier status, and their hemophiliac relatives, were studied. Five of the 8 had a family history of hemophilia, and carrier status was confirmed by FIX gene restriction fragment length polymorphism (RFLP) linkage analysis. The remaining 3 had a history compatible with sporadic hemophilia, and carrier status could not be excluded by linkage analysis. The factor IX gene mutation was unknown in each pedigree prior to these analyses.

PCR and HD

FIX gene sequences representing a 176-bp 5′ region and the eight exons and intron borders were PCR-amplified in size ranges of 197–721 bp using AmpliTaq (Cetus Perkin-Elmer, Norwalk, CT). Primers were synthesized according to the FIX gene sequence of Yoshitake et al. [1]: 5′ and exon 1, sense (s) −176 to antisense (a) 151 (327 bp); exons 2–3, s6012–a6733 (721 bp); exon 4, s10343–a10547 (204 bp); exon 5, s17627–a17937 (310 bp); exon 6, s20316–a20666 (350 bp); exon 7, s30003–a30200 (197 bp); exon 8, 5′ s30779–a31079 (300 bp); 3′ s31079–a31399 (320 bp). PCR conditions included 30 cycles at 94°C × 30 sec, annealing at 52°C × 30 sec, and 72°C × sec. Five–10 μl of the PCR reaction was electrophoresed in 1-mm-thick, 40 × 20 cm Hydrolink-MDE polymer gels (AT Biochem, Malvern, PA) at 500 volts for 5–10 hr in 0.6 × tris borate disodium ethylene

Received for publication October 20, 1995; accepted November 8, 1995.

Address reprint requests to M. Catherine Driscoll, M.D., now at the Children's National Medical Center, Department of Hematology-Oncology, 111 Michigan Avenue NW, Washington, DC 20010.

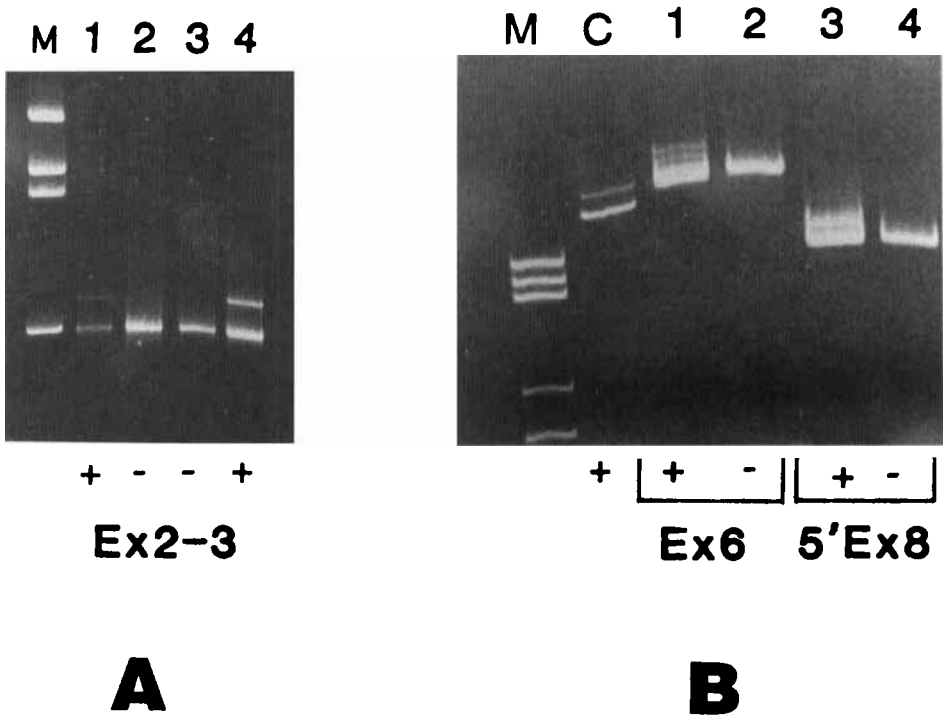

Fig. 1. Heteroduplex analysis of PCR-amplified FIX gene. A: FIX exons 2–3. Lane M, ϕ*xhae*II size marker; lane 1, hemophilia B carrier (codon 37$^{ins'A'}$); lane 2, normal male; lane 3, hemophilia male (codon 37$^{ins'A'}$); lane 4, mixture of DNA from normal (lane 2) and hemophilia B (lane 3) males. +, positive for HD; −, negative for HD. B: FIX exons 6 and 5′ 8. Lane M, ϕ*xhae*II size marker; lane C, control HD (AT Biochem); lane 1, hemophilia B carrier (codon 180$^{C\rightarrow T}$); lane 2, hemophilia B male (codon 180$^{C\rightarrow T}$); lane 3, hemophilia B carrier (codon 272$^{C\rightarrow T}$); lane 4, hemophilia B male (codon 272$^{C\rightarrow T}$). +, positive for HD; −, negative for HD.

diamine tetraacetate (TBE) buffer. The gel was then stained with ethidium bromide (1 μg/ml) and photographed. PCR samples of females were analyzed directly without further manipulation. PCR samples from hemophiliac males were mixed with a sample from a normal male, heated at 94°C for 5 min, and cooled to room temperature over 1 hr prior to electrophoresis.

DNA Sequence Analysis

Double-stranded PCR-generated DNA was isolated from a low-melting point agarose gel by the GeneClean glass bead method (Bio 101, La Jolla, CA) and was sequenced by the Sanger di-deoxy method (Circumvent, New England Biolabs, Beverly, MA) using gamma [^{32}P] ATP end-labeled amplification primers.

FIX Haplotypes

Analysis of FIX gene RFLPs *DdeI*/IVS1, *TaqI*/IVS5, and *MnlI*/exon 6 was performed by PCR according to Green et al. [7].

RESULTS

Heteroduplexes were detected in each of the eight female DNA samples. Typical HDs are shown in Figure 1. Two novel and six recurrent FIX gene mutations were identified (Table I). The two unique mutants included: 1) a single base pair insertion of adenine in codon 37 in exon 2 (nucleotide 6484) resulting in a frameshift and formation of a premature termination at codon 40, and 2) a codon 272 C → T transition in exon 8 (nucleotide 30935) resulting in a Leu → Phe missense mutant (Fig. 2). Since codon 272 is a partially generic amino acid residue, identical in all species of FIX and in factor VII and protein C, the missense substitution should represent a causative hemophilia mutation [8]. The six recurrent mutants included four at CpG dinucleotides: an identical 180 C → T (Arg → Trp) missense in two unrelated pedigrees with different FIX haplotypes (Table I, pedigrees 2 and 3), and codon 252 C → T and codon 338 C → T transitions resulting in nonsense codons. The two non-CpG repeat mutants resulted in missenses: codon 289 G → T (Cys → Phe) and 336 G → A (Cys → Tyr). Codons 180, 289, and 336 each code for a generic FIX amino acid and, as such, represent residues important for coagulant protein function [8].

DISCUSSION

X-linked disorders, such as hemophilia B, are due to new mutations in approximately one third of cases, mak-

TABLE I. Mutations in Hemophilia B Patients With Heteroduplexes*

Pedigree no.	1	2	3	4	5	6	7	8
FIXC	<1%	<1%	<1%	<1%	<1%	<1%	<1%	<1%
Haplotype								
DdeI	−	−	+	−	−	−	−	−
TaqI	+	+	−	−	+	+	+	−
MnlI	+	+	−	−	+	+	+	−
Inheritance	FH	FH	FH	FH	FH	Sp	Sp	Sp
FIX mutant	$37^{ins'A'}$	$180^{C\to T}$	$180^{C\to T}$	$252^{C\to T}$	$272^{C\to T}$	$289^{G\to T}$	$336^{G\to A}$	$338^{C\to T}$
Exon	2	6	6	8	8	8	8	8
Nucleotide	6484	20518	20518	30875	30935	30987	31128	31133
Amino acid	FS	Arg→Trp	Arg→Trp	STOP	Leu→Phe	Cys→Phe	Cys→Tyr	STOP

*FH, family history; Sp, sporadic; FS, frameshift; STOP, nonsense codon.

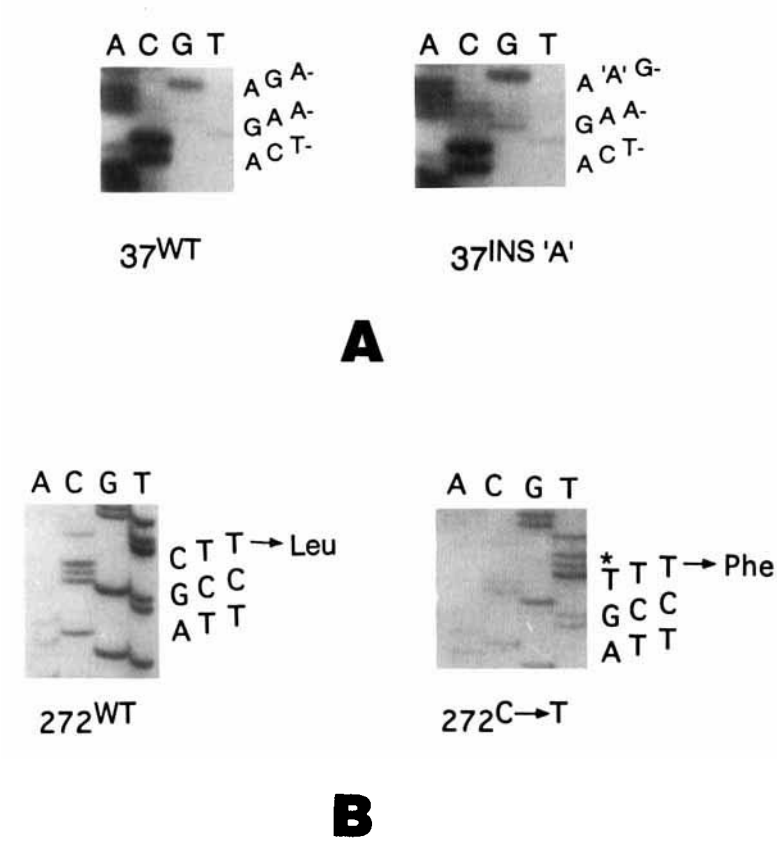

Fig. 2. Direct DNA sequence analysis. A: Codon 37_{wt}, normal wild-type sequence; codon $37^{ins'A'}$, mutant sequence with insertion of single base pair, adenine, producing a frameshift. B: Codon 272^{wt}, normal wild-type sequence; codon $272^{C\to T}$, mutation producing a Leu → Phe missense substitution.

ing carrier studies and prenatal diagnosis especially problematic. Three of eight hemophilia carriers in this study could not be diagnosed by conventional linkage analysis. HD analysis thus improves on the accuracy and availability of DNA diagnostics in hemophilia. HD analysis is a simple screening method with a sensitivity on the order of the 85–95% reported for SSCP and DDGE, limited only by the length of the PCR product and the site of the mutation [6,9].

ACKNOWLEDGMENTS

This work was supported by HL38265 (NYH-Cornell Medical Center) (M.C.D.) and by the Children's Blood Foundation.

REFERENCES

1. Yoshitake S, Shach BG, Foster DC, Davie EW, Kurachi K: Nucleotide sequence of the gene for human factor IX (antihemophiliac factor B). Biochemistry 24:3736, 1985.

2. Giannelli F, Green PM, Sommer SS, Lillicrap DP, Ludwig M, Schwaab R, Reitsma PH, Goossens M, Yoshioka A, Brownlee GG: Haemophilia B: Database of point mutations and short additions and deletions, fifth edition, 1994. Nucleic Acids Res 17:3534, 1994.
3. Fischer SG, Lerman LS: DNA differing by a single base-pair substitution are separated in denaturing gradient gels. Correspondence with melting theory. Proc Natl Acad Sci USA 80:1579, 1983.
4. Keen J, Lester D, Inglehearn C, Curtis A, Bhattacharya S: Rapid detection of single base mismatches as heteroduplexes on Hydrolink gels. Trends Genet 7:5, 1991.
5. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 86:2766, 1989.
6. Chen SH, Schoof JM, Weinmann AF, Thompson AR: Heteroduplex screening for molecular defects in factor IX genes from hemophilia B families. Br J Haematol 89:409, 1995.
7. Green PM, Montandon AJ, Ljung R, Nilsson IM, Giannelli F: Haplotype analysis of identical factor IX mutants using PCR. Thromb Haemost 67:66, 1992.
8. Bottema CDK, Ketterling R, Li S, Yoon HS, Phillips JA, Sommer SS: Missense mutations and evolutionary conservation of amino acids: Evidence that many of the amino acids in factor IX function as "spacer" elements. Am J Hum Genet 49:820, 1991.
9. White MB, Carvallo M, Derse D, O'Brien SJ, Deam M: Detecting single base substitutions as heteroduplex polymorphisms. Genomics 12:301, 1992.